IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

CAROLINE S. BROWN 1813 ART UNIT: 1813

SERIAL NO. : 08/465,747 EXAMINER: Mosher

FILED : June 6, 1995

FOR : HUMAN PARVOVIRUS B19 PROTEINS AND VIRUS-LIKE PARTICLES, THEIR PRODUCTION AND THEIR USE IN DIAGNOSTIC ASSAYS AND VACCINES

DECLARATION OF DR. WILLY J.M. SPAAN

Hon Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Willy J.M. Spaan, do hereby declare:

1. I am currently a full professor and head of the Department of Virology, and Chairman of the Institute of Medical Microbiology, Medical Faculty, at State University of Leiden, The Netherlands. Attached hereto is a copy of my Curriculum Vitae, which sets forth my academic and scientific credentials.

2. I have read and am familiar with the above application. I have also read the Information Disclosure Statement And Second Preliminary Amendment filed in that application, and I am in agreement with the position taken in that document that claims 49-57 of the above application would not have been obvious to one skilled in the art as of September 14, 1989.

3. While Ozawa et al., J. Virol, 61(8): 2395-2406 (1987) identified the VP1 and VP2 capsid proteins of the human parvovirus B19 in infected human erythroid bone marrow cells, they did not disclose isolated and purified VP1 and VP2 capsid protein as defined in claims 49-57 of the above application.

4. As described in Cotmore et al., J. Virol, 60(2): 548-557 (1986), page 555, left column, Cotmore et al. expressed in E. Coli. a "tripartite fusion protein" and a "bipartite fusion protein" each containing a B19 capsid protein fragment consisting of 284 amino acids of the VP1 capsid protein, namely amino acids 151-435 of VP1. See the attached diagram. As shown in the attached diagram, in the "tripartite fusion protein", the B19 fragment was fused between lambda repressor protein and beta-galactosidase whereas in the bipartite fusion protein, the beta-galactosidase was deleted. In both fusion proteins, however, the B19 fragment was just that, a mere fragment of a B19 capsid protein. Cotmore et al. thus failed to express any fused, complete VP1 or VP2 capsid protein. Cotmore et al. certainly failed to produce any unfused VP1 or VP2 capsid protein.

5. Sisk et al., Bio/Technology, 5: 1077-1088 (1987) also expressed a fusion protein in E. coli. Sisk et al.'s fusion protein (196 KD) consisted of beta-galactosidase and a polypeptide encoded by the structural gene encoding capsid proteins VP1 and VP2. This fusion protein was stated to be recognized by B19 antibodies present in B19 positive sera, but yet the fusion protein detected antibodies in only 22 out of the 50 sera (44%) obtained from normal healthy adults. Sisk et al. surmise, page 1079, right column, first sentence under "DISCUSSION", that their beta-galactosidase - B19 fusion protein was "a reliable source of viral antigen for detection of B19 related antibodies in human serum". In my opinion, those skilled in the art would not readily agree.

6. It is significant that neither Sisk et al. nor Cotmore et al. state that their fusion proteins had the native conformation of the B19 capsid. While the fusion proteins could possibly be of limited value in a Western blot analysis, which denatures the protein and exposes epitopes, they would not be of value in a more sensitive type of assay, such as ELISA, where the three-dimensional structure of the protein must be preserved. In my opinion the skilled worker would know that the fusion partner would most likely prevent the fusion protein from folding to the precise structure of the B19 capsid protein. The skilled worker would thus have cause to doubt whether B19 capsid proteins produced as fusion proteins would have all of the native conformational epitopes of the B19 capsid.

7. Collett et al., Reviews In Medical Virology, 4: 91-103 (1944), confirms my opinion. Thus, Collett et al. explains that recombinant fusion proteins expressed in E. coli lack the native conformation of the B19 capsid. See page 98, left column. Mentioned among these fusion proteins are those of Cotmore et al. and Sisk et al. In my opinion, the skilled worker would thus have cause to doubt whether such fusion proteins would reliably detect B19 antibodies in human sera.

8. In fact, the Sisk et al. fusion protein would have been recognized by those skilled in the art as giving rise to numerous false negatives. It is known that about 70-80% of human sera contain B19 antibodies. See, e.g., Cohen et al., J. Med. Microbiol., 25: 151-153 (1988) and Gay et al., Communicable Disease Report, 4(a): R104-R107 (1994), copies enclosed. See

also page 15, lines 32-36 of the above application where it is reported that 76% of randomly selected donors were positive. Since the Sisk et al. fusion protein gave only 44% positives, in my opinion those skilled in the art would have known that these fusion proteins failed to detect a large percentage of positives.

9. Since Cotmore et al., provided no data in detection of B19 antibodies in human sera, in my opinion their work does not survive more than minimal scrutiny.

10. In my opinion, it is significant that the VP1 and VP2 capsid proteins of the invention provide a diagnostic agent for detecting B19 antibodies in human sera that gives no false positives and no false negatives. The data in Tables 1 and 2 of the above application show that the VP1 and VP2 capsid proteins of the invention gave no false positives and no false negatives when used to detect B19 antibodies in human sera. See also, the immunofluorescence studies reported in Brown et al., Virus Res., 15: 197-212 (1990), which likewise show no false negatives or false positives. It is surely a principle of diagnostic assays that the ideal assay gives no false positives and no false negatives. Given the state of the prior art, in my opinion, it was surprising and unexpected that the VP1 and VP2 capsid proteins of the invention could provide this highly advantageous result.

11. Wood et al. U.S. Patent 4,971,793 and Mazzarra et al. (WO 8802026) are not relevant to claims 49-57 because canine parvovirus and human B19 parvovirus are structurally quite dissimilar. Chapman et al., Virology, 194, 491-508 (1993),

discusses an alignment of parvovirus capsid sequences against the sequence and three-dimensional structure of CPV. The sequence similarity between CPV and B19 was highest for the conserved structural motif which forms the inner core of the particle. The overall identity was on the icosahedral three fold axes, which contain residues involved in host range and antigenicity. Regarding the surface features there was a close but inexact correspondence of B19 antigenicity and CPV surface accessibility suggesting that the general region is antigenic but that the structures are likely to differ in detail.

12. Agbande et al., Virology, 203, 106-115 (1994), describes the structure of baculovirus-produced VP2 capsids at a fairly low resolution, 8 Angstroms. This means that only very large structural features can be distinguished, such as the inner core of the particle. To obtain more information, the B19 structure map was compared to that of the high resolution map of feline parvovirus (FPV) to which CPV is almost identical, which demonstrated the similarity between these inner core structures. However, in my opinion this similarity is unimportant because the inner core structure is conserved in most icosahedral viruses infecting eukaryotes, and it is the surface features that are important in a diagnostic assay, since the epitopes are on the surface. The comparison between B19 and FPV showed striking differences in the surface topology as, in the B19 capsids, the prominent three fold spikes were absent (see the gap in the sequence alignment of Fig. 6 between residues 415-435; this figure was adapted from the alignment of Chapman et al., Fig. 3;

see also Fig. 8 in the Agbandje paper). Other surface differences which were difficult to interpret have also been predicted by Chapman et al. (insertions and deletions in the sequence alignment).

13. Cotmore et al. also notes at page 548, right column, that

> B19 is a parvovirus, but DNA hybridization studies have showed that it is only very distantly related to other serotypes in this family.

citing Cotmore et al., Science, 226: 1161-1165 (1984), while Cossart et al., The Lancet, January 11, 1995, 72-73, show that canine parvovirus and human B19 parvovirus do not cross-react antigenically.

14. In my opinion, since there is no cross-reaction antigenically between canine parvovirus and human B19 parvovirus and there is low sequence homology and low DNA homology between canine parvovirus and human B19 parvovirus, the skilled worker would have had no basis to extrapolate the work done by Wood et al. and Mazzarra et al. on the use of CPV as a vaccine to the expectation that B19 capsid proteins would be useful as vaccines, much less that they would be useful as diagnostic agents for reliably detecting B19 antibodies in human sera.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dr. Willy J.M. Spaan

Dated:

September 28, 1995

June 30, 1995

CURRICULUM VITAE

NAME	:	Wilhelmus Josephus Maria Spaan
DATE & PLACE OF BIRTH	:	August 18, 1954, Geleen (NL)
NATIONALITY	:	Dutch
SEX	:	Male
MARTIAL STATUS	:	Married, four children
ADDRESS	:	Jacoba van Beierenweg 7 2215 KS Voorhout

EDUCATION:

1966-1973 High School	:	"Albert Schweitzer" Atheneum-B, Geleen, The Netherlands
1973-1980 University	:	Biology study; State University Utrecht, The Netherlands
1980	:	Doctoral exam (cum laude)
1980-1984	:	PhD study on the replication strategy of coronaviruses
1984	:	PhD thesis (cum laude), Veterinary Faculty, Utrecht, The Netherlands

SCIENTIFIC APPOINTMENTS:

1981-1984	:	ZWO Research Associate at the Institute of Virology (chairman Prof.Dr. M.C. Horzinek, Veterinary Faculty, State University Utrecht, The Netherlands.
1984	:	Scientific Staff Member, Institute of Virology (chairman Prof.Dr. M.C. Horzinek), Veterinary Faculty, State University Utrecht, The Netherlands
1984-1985	:	Fullbright fellow at the Salk Institute, Dept. of Molecular Biology and Virology, La Jolla, USA
1986-1989	:	Head of the Section Molecular Virology of the Department of Infectious Diseases and Immunology (chairman Prof.Dr. M.C. Horzinek), Veterinary Faculty, State University Utrecht, The Netherlands
1990-present	:	Full professor and head of the Department of Virology; Chairman of the Institute of Medical Microbiology, Medical faculty, State University of Leiden, The Netherlands.

PROFESSIONAL INSTITUTIONS:

Member of the Coronaviridae, Togaviridae and Toroviridae study groups of the Vertebrate Virus Subcommittee of the International Committee on the Taxonomy of Viruses.

Vice-chairman of the section 'Persistent virus infections and oncogenic transformation' of the NWO-Foundation for Medical and Health Research.

Member of the advisory committee on genetic modification of the Dutch Ministry of

Health and Environmental Hygiene.

Chairman of the Board of the section 'Nucleic acids' of the NWO-Foundation for Chemical Research.

Member of the Board of the Medical Research Council

EDITORIAL POSITIONS:

- Member of the Editorial Board of the Journal of General Virology, (1990-1994)
- Ad hoc reviewer for Virology
- Ad hoc reviewer for Journal of Virology
- Ad hoc reviewer for BBA
- Ad hoc reviewer for Gene

SERVICE ON NATIONAL AND INTERNATIONAL GRANT REVIEW PANELS, STUDY SECTIONS:

Member of the grant committee of Genetics and Virology of the NWO-Foundation for Medical and Health Research

Chairman of the NWO-AIDS-research grant committee

Ad hoc grant reviewer, National Science Foundation

Ad hoc grant reviewer, National Institute of Health

Ad hoc grant reviewer, Swiss National Science Foundation

SERVICES ON UNIVERSITY OF UTRECHT, FACULTY OF VETERINARY MEDICINE:

1987 - 1990	Member executive board of the Department of Infectious Diseases and Immunology

SERVICE ON UNIVERSITY OF LEIDEN, FACULTY OF MEDICINE:

1990 - 1993	Member Advisory Committee undergraduate Program Biomedical Sciences
1991 - 1994	Member executive Board "Boerhave" Commission for Post-academic training program
1991-present	Chairman of the Institute of Medical Microbiology, Medical Faculty State University of Leiden, The Netherlands.
1993-present	Member executive Board, School of Medicine, Leiden University

SERVICES ON ACADEMIC HOSPITAL:

1990-present	Member of management team of Division 4
1991-present	Biosafety officer

SERVICES ON COMMUNITY:

1993-present	Member of the "Curatorium" Rijnlands Lyceum Sassenheim

SUPERVISOR PhD STUDENTS

- de Groot, R.J., *A molecular study of feline infectious peritonitis virus,* Utrecht, Diss., 1988
- Luytjes, W., *Surface proteins of murine and bovine coronaviruses,* Utrecht, Diss., 1989
- Bredenbeek, P.J., *Nucleic acid domains and proteins involved in the replication of coronaviruses,* Utrecht, Diss., 1990
- Snijder, E.J., *Berne virus: Replication and evolution of the torovirus prototype,* Utrecht, Diss., 1991
- Vennema, H., *The proteins of feline infectious peritonitis coronavirus: their biosynthesis and involvement in pathogenesis,* Utrecht, Diss., 1991
- Beersma, M.F.C., *The interaction of cytomegalovirus with HLA class I molecules,* Amsterdam, Diss., 1993
- Brown, C.S., *Human Parvovirus B 19 expression and applications of its structural proteins,* Leiden, Diss., 1994
- Most, R.G. van der, *Coronavirus defective interfering RNAs: A tool to study replication, transcription and recombination,* Leiden, Diss., 1994.

OTHER SCIENTIFIC ACTIVITIES

- Co-organizer EMBO workshop on Molecular Biology and Pathogenesis of Coronaviruses, Utrecht, 1093.
- Co-organizer Third International Congress on Positive Stranded RNA Viruses, Tampa, USA, 1992.
- Organizer Fourth International Congress on Positive Stranded RNA viruses, Utrecht, 1995.

GRANT SUPPORT

1. *Production of recombinant coronavirus subunit vaccines,* Duphar BV Weesp, the Netherlands, 1986-1988, 1 post-doc, 1 PhD student, 1 technician.

2. *Development of viral vaccines,* Duphar B.V. Weesp, the Netherlands, 1989-1993, 2 post-docs, 1 PhD student, 2 technicians.

3. *Discontinuous transcription of coronavirus mRNAs,* Dutch Foundation for Chemical Research, 1989-1993, 1PhD student.

4. *The role of the spike protein in coronavirus pathogenesis,* Dutch Foundation for

Medical Research, 1989-1992, 1 PhD student.

5. *Cell-mediated immune response against murine coronaviruses,* Dutch Foundation for Medical Research, 1990-1994, 1 PhD student, 1 technician.

6. *The interaction of coronavirus surface proteins with the receptor,* Dutch Foundation for Medical Research, 1992-1995, 1 PhD student.

7. *Molecular approaches to the control of coronavirus-associated disease,* European community Science Program, 1992-1995, 1 post-doc.

8. *Coronavirus RNA transcription,* Dutch Foundation for Chemical Research, 1993-1997, 1 PhD student.

9. *The role of RNA-protein and protein-interactions in the assembly of hepatitis C virus,* Dutch Foundation for Medical Research, 1995-1998, 1 post-doc.

10. *Coronavirus RNA encapsidation,* Dutch Foundation for Chemical Research, 1995-1999, 1 PhD student.

INVITED SEMINARS OR LECTURES 1985- present

- *Replication strategy of coronaviruses,* Department of Molecular Biology and Virology, Salk Institute, La Jolla USA, March 1985

- *Coronavirus mRNA synthesis,* Department of Neurology and Microbiology, School of Medicine, Los Angelos, USA, April 1985

- *Replication strategy of coronaviruses,* Department of Pathology, Uniformed Health Services, Bethesda USA, May 1985

- *Replication strategy of coronaviruses,* Department of Microbiology, School of Medicine, Philadelphia, USA, May 1985

- *Replication of coronaviruses: an overview,* Division of Biology, Caltech, Pasadena USA, September 1986

- *Replication of coronaviruses: an overview,* Cell Biology, EMBL, Heidelberg, Germany, November 1986

- *Structural proteins of coronaviruses,* Wadsworth Center for laboratories and Research, Albany, USA, September 1989

- *Structural proteins of coronaviruses,* Department of Microbiology, Mount Sinaï School of Medicine, New York, USA, March 1988

- *Early death after challenge with feline infectious peritonitis virus of kittens immunized with a recombinant vacciniavirus expressing the FIPV spike protein,* Cold Spring Harbor Conference on Modern approaches to new vaccines including AIDS, September 1989

- *Coronaviruses: Genome organization and expression,* Centro de Biologia Molecular,

Madrid, Spain, November 1989

- *Comparative and evolutionary aspects of coronaviral, arteriviral and toroviral genome structure and expression,* 2nd International symposium on positive stranded RNA viruses, Vienna, Austria, June 1989

- *Equine arteritis virus, Genome organisation and expression*, Workshop Positive stranded RNA viruses, Anual meeting of the Society for General Microbiology, Warwick, UK, April 1990

- *Equine arteritis virus: Genome structure, expression and evolutionary aspects,* 9th annual meeting of American Society of Virology, Salt Lake City, USA, July 1990

- *Equine arteritis virus: Genome structure, expression and evolutionary aspects*, Department of Molecular Microbiology, School of Medicine, Washington University, St. Louis, USA, July 1990

- *Equine arteritis virus: Genome structure, expression and evolutionary aspects,* Department of Biology, Georgia State University, Atlanta, USA, July 1990

- *The coronavirus superfamily,* Department of Micriobiology, University of Minnesota, Minneapolis, USA, Januari 1991

- *The coronavirus superfamily,* Center of Disease Control, Atlanta, USA, Januari 1991

- *Molecular Biology of Coronaviruses,* Institute of Virology and Immunology, Würzburg, Germany, December 1991

- *Molecular Biology of Coronaviruses,* Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany, December 1991

- *Site-directed mutagenesis of a coronavirus genome using homologous recombination,* Workshop Positive stranded RNA viruses, Anual meeting of the Society of General Micriobiology, Cardiff, March 1992

- *Homologous RNA recombination allows efficient introduction of site-specific mutations into the genome of coronavirus MHV-A59 via synthetic co-replicating RNAs,* Third International Positive Stranded RNA virus conference, Tampa, USA, September 1992

- *Some aspects of the replication of coronaviruses,* Institute of Virology, Marburg, Germany, January 1993

- *Coronavirus DI RNAS: a tool to study replication, transcription and recombination,* Keystone Symposium on Molecular biology of humanpathogenic viruses, Lake Tahoe, March 1993

- *Some aspects of the replication of coronaviruses,* Scripps Research Institute, La Jolla, USA, March 1993

- *Leader - primed transcription of Coronaviruses,* IXth International congress of Virology, Glascow UK, August 1993

- *Coronavirus DI RNAs: A tool to study replication transcription and recombination,*

Workshop Genetic recombination and defective interfering particles in RNA virus, Madrid, maart 1994.

- *Towards the development of a coronavirus expression vector,* Workshop Resistance to viral infection, Madrid, juni 1994

- *Common and distinctive features of Corona-, Toro- and Arteriviruses: molecular and biological Aspects,* 6th International Symposium on Corona and related viruses, Quebec, 27-8/1-9, 1994

- *Open reading frame in DI amplification,* 6th International Symposium on Corona and related viruses, Quebec, 27-8/1-9, 1994.

- *Corona- and arteriviruses: genome expression and replication,* SGM Meeting Leeuwenhorst September 1994.

Bibliography

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Spaan, W., Luytjes, W., Geerts, D., and Koolen, M.(1988). The function of the coronavirus spike protein in viral pathogenesis. *J. Cell Biochem.* **suppl. 12C**, 38

Vennema, H., de Groot, R., Dalderup, M., and Spaan, W.(1988). Expression studies for coronavirus subunit vaccine development. *J. Cell Biochem.* **suppl. 12B**, 23

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Pachuk, C. J., Bredenbeek, P. J., Zoltick, P. W., Spaan, W. J. M., and Weiss, S. R.(1989). Molecular cloning of the gene encoding the putative polymerase of mouse hepatitis coronavirus, strain A59. *Virology* **171**, 141-148.

Snijder, E. J., Den Boon, J. A., Spaan, W. J. M., Verjans, G. M. G. M., and Horzinek, M. C.(1989). Identification and primary stucture of the gene encoding the berne virus nucleocapsid protein. *J. Gen. Virol.* **70**, 3363-3370.

Spaan, W. J. M., Vennema, H., de Groot, R., Dalderup, M., Horzinek, M. C., Harbour, I., and Gruffydd-Jones, T.(1989). *Vaccines '89: Modern approaches to new vaccines including prevention of aids* (Lerner, R. A., Ginsberg, H., Chanock, R. M., and Brown, F. Eds.) Cold Spring Harbor Laboratory, New York. 491-496.

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R., and Spaan, W. J. M.(1990). The Primary Structure and Expression of the 2nd Open Reading Frame of the Polymerase Gene of the Coronavirus MHV- A59 - A Highly Conserved Polymerase Is Expressed by an Efficient Ribosomal Frameshifting Mechanism. *Nucleic Acids Res.* **18**, 1825-1832.

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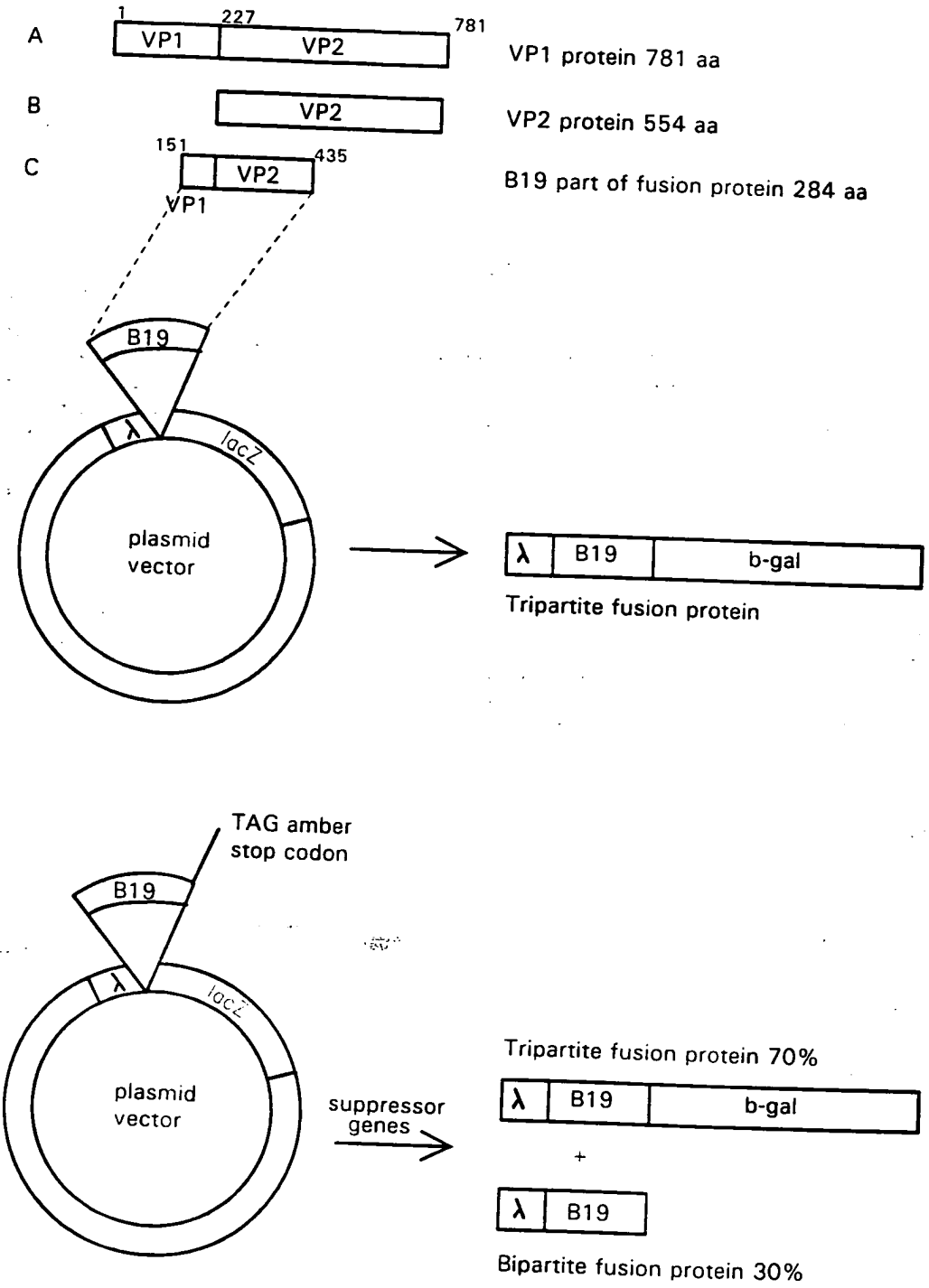

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J. Med. Microbiol. — Vol. 25 (1988), 151–153

SHORT ARTICLE

The prevalence of antibody to human parvovirus B19 in England and Wales

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Summary. The prevalence of antibody to human parvovirus B19 (anti-B19 IgG) in England and Wales was measured by an antibody-capture radioimmunoassay. Over 2000 sera were examined; 1422 from the general population, 374 from unselected children admitted to hospital and 300 from women attending an antenatal clinic. Waning levels of maternally-derived antibody were found in infants under 1 year old. In children 1–5 years old, 5–15% had anti-B19 IgG and this rose to 50–60% in older children, young adults and women of child-bearing age. In older people, the prevalence of anti-B19 IgG increased with age, rising to more than 85% in those over 70 years old.

Introduction

The prevalence of antibody to human parvovirus B19 (anti-B19) in different age groups of the general population has been measured in Japan (Nunoue *et al.*, 1985), the USA (Anderson *et al.*, 1986) and West Germany (Schwarz *et al.*, 1987). In the United Kingdom, the prevalence has been analysed in children but not, except in outline, in adults (Cossart *et al.*, 1975; Edwards *et al.*, 1981). We now report on the prevalence of anti-B19 in all age groups of the general population in England and Wales. In the previous studies, the techniques used were counter-immunoelectrophoresis or enzyme-linked immunosorbent assay; we used antibody-capture radioimmunoassay (RIA) (Cohen *et al.*, 1983).

Materials and methods

Test sera

Three series of sera were examined:

(1) *General population, England and Wales, 1985 and 1986.* Sera from 1422 unselected patients, collected for an influenza survey during the summers of 1985 (676 sera) and 1986 (746 sera), were obtained from the Public Health Laboratories in Taunton, Exeter, Birmingham, Nottingham, Leicester, Peterborough, Rhyl and Preston and were made available to us by Dr P. Chakraverty of the Virus Reference Laboratory.

(2) *Children, London, 1982 and 1983.* Sera from 374 unselected children (< 14 years old) admitted to a North London hospital between March 1982 and September 1983 were kindly provided by Dr J. Reeve, Prince of Wales Hospital, London N15.

(3) *Women of child-bearing age, London, 1985 and 1986.* Sera from 300 women attending an antenatal clinic in North West London, collected for influenza studies during November 1985 (150 sera) and March 1986 (150 sera), were made available by Dr P. Chakraverty.

Received 9 July 1987; accepted 14 July 1987.

Assay for antibody to human parvovirus

Immunoglobulin G class antibodies to human parvovirus B19 were detected by antibody-capture RIA (Cohen *et al.*, 1983).

Results

The prevalence of anti-B19 IgG in the different age groups of the population of England and Wales was very similar over the 2 years sampled, 1985 and 1986. The combined results for the 2 years are given in the table.

The prevalence of anti-B19 IgG in children in London during 1982–83 is shown in the figure.

Out of 150 antenatal sera tested for each year, 86 (57%) were anti-B19 IgG positive in 1985 and 75 (50%) in 1986. Overall, 161 out of 300 (53%) test sera gave positive results.

Discussion

The prevalence of anti-B19 IgG in infants under 1 year old reflects waning levels of maternally-derived antibody. The results from children show that active infection with B19 virus occurs fre-

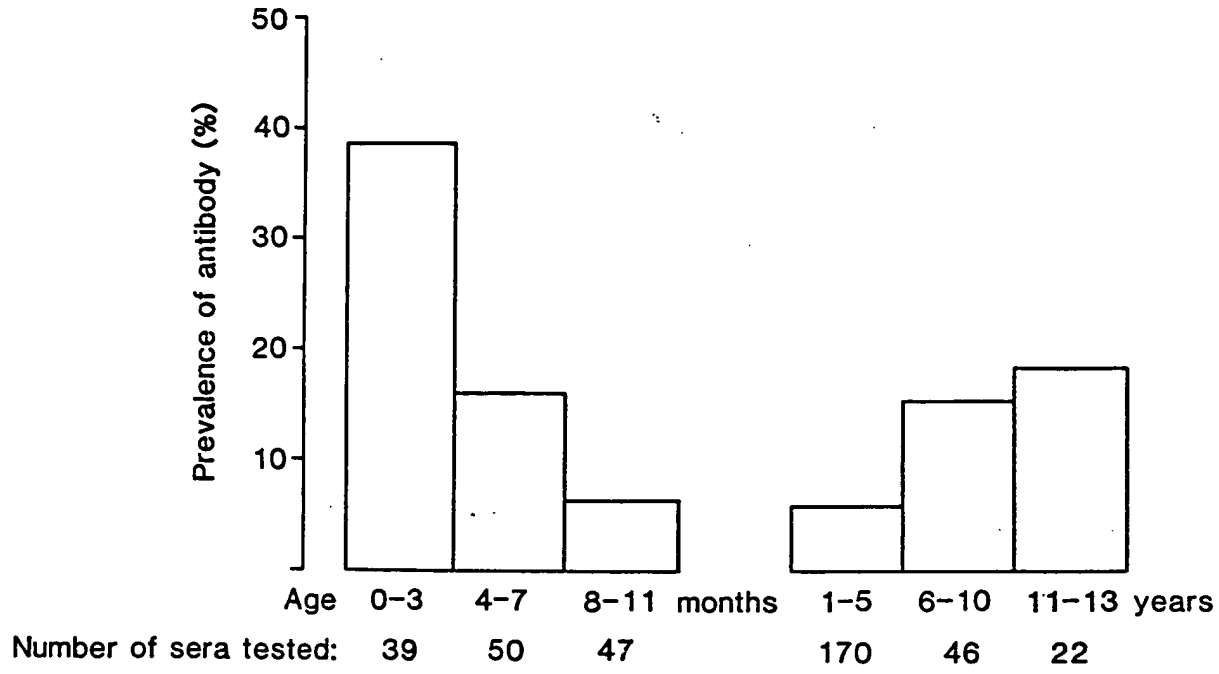

Figure. Prevalence of anti-B19 IgG in children admitted to a North London hospital in 1982–83.

Table. Prevalence of antibody to parvovirus B19: 1985–86

Age (years)	Number of sera tested	Number (%) of positive results
<1	33	18 (54·5)
1–5	160	24 (15·0)
6–10	117	42 (35·9)
11–15	106	55 (51·9)
16–20	135	81 (60·0)
21–30	281	145 (51·6)
31–40	162	92 (56·8)
41–50	105	80 (76·2)
51–60	73	58 (79·5)
61–70	97	71 (73·2)
>71	153	133 (86·9)

quently in childhood, as originally demonstrated by Cossart *et al.* (1975) and Edwards *et al.* (1981) and later confirmed by Anderson *et al.* (1984) when investigating the aetiology of erythema infectiosum.

The frequency of antibody in children was lower in North London in 1982–83 than in the general population in 1985–86. It may be significant that most of the sera from the North London children were collected before May 1983 when there was a large outbreak of erythema infectiosum (Anderson *et al.*, 1984).

In adults aged 16–40 years, 50–60% had antibody. This is similar to the 61% prevalence previously found in blood donors by the same assay method (Cohen *et al.*, 1983). Almost half the women of child-bearing age are, therefore, susceptible to B19 virus with the attendant risk of fetal loss should infection occur in pregnancy (Carrington *et al.*, 1987). The prevalence of antibody rises after 40 years of age, suggesting continuing exposure to the virus in this age group.

The prevalence of anti-B19 antibody in this study is higher than in other surveys. This is probably attributable to the greater sensitivity of the RIA method.

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Age specific antibody prevalence to parvovirus B19: how many women are infected in pregnancy?

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Summary

Infection with parvovirus B19 is an important cause of late fetal mortality in the second trimester, and many infections in pregnancy remain undiagnosed. A serological survey stratified by age has been used to estimate the incidence of maternal infection with parvovirus B19 in pregnancy. Serum remaining from specimens submitted for diagnosis from 6864 people of all ages to seven public health laboratories in England was tested for antibody to parvovirus B19. The antibody prevalence rose with age to 45% at 10 years and 60% to 70% in adults. The age specific force of infection was highest in children aged less than 10 years and lowest in adults. Maternal infection with parvovirus B19 is estimated to occur in approximately one pregnancy in 400. It has been estimated that fetal death occurs in 9% of these cases, which suggests that parvovirus B19 may cause more than 150 fetal deaths in England and Wales each year. Testing for evidence of recent infection with parvovirus B19 should be considered for unexplained cases of fetal hydrops in the second trimester, especially in years of parvovirus B19 epidemics.

Introduction

Parvovirus B19 causes erythema infectiosum[1], commonly known as slapped cheek syndrome or fifth disease. It is clinically similar to rubella and the diseases can be distinguished reliably only by laboratory tests. The disease is usually mild, but infection during pregnancy has been estimated to cause fetal death in about 9% of cases[2]. There is no evidence to suggest that parvovirus B19 is associated with congenital abnormality. A serological survey was performed in 1991 and the results, stratified by age, were analysed to estimate the incidence of parvovirus B19 infection in pregnant women.

Methods

Laboratory methods

Serum remaining from specimens submitted from patients of all ages for routine diagnostic examination to seven public health laboratories (Ashford, Birmingham, Exeter, Leeds, Manchester, Preston, and Reading) during 1991 was tested. Specimens from immunocompromised patients and samples sent for testing for hepatitis B virus and antibodies to the human immunodeficiency virus were excluded.

The serum specimens were tested for anti-B19 IgG using an enzyme linked immunosorbent assay (ELISA) (MRL Diagnostics, Cypress, California) at Preston Public Health Laboratory. Preliminary studies at the Virus Reference Division at the PHLS Central Public Health Laboratory had shown that the sensitivity and specificity of the ELISA was similar to that of antibody capture radioimmunoassay for anti-B19 IgG[3]. Results were expressed as an index calculated as the ratio of the optical densities of test specimens to a reference specimen provided with the ELISA kit. The kit manufacturer recommends that specimens with index values greater than 1.2 be considered seropositive; those between 0.8 and 1.2, equivocal; and those less than 0.8, seronegative.

In a serological survey it is essential to ensure that the correct proportions of specimens are classified as positive or negative in each age group, but not that every individual specimen is correctly classified as positive or negative. A cut off point should be chosen to balance false negatives against false positives. Force of infection estimates were derived using cut off ratios of 0.8, 0.9, and 1.0: ratios greater than the cut off were classified as seropositive and values less than the cut off as seronegative. A cut off of 1.0 divides the equivocal range equally between positive and negative, whereas a cut off of 0.8 assigns all equivocal results as positive, which may be more appropriate if the test is insensitive.

Epidemiological methods

The force of infection, λ, is defined as the rate at which susceptible people acquire infection[4]. The probability that a susceptible person will be infected in a short period of time is the product of the force of infection and time. The force of infection changes in the course of an epidemic cycle: it is greater at the height of an epidemic. We calculate its mean value, averaged over epidemic and non-epidemic periods. The force of infection varies with age for most directly transmitted diseases, and is typically highest in primary school children and lowest in adults. The age specific force of infection, (a), is related to the prevalence at age a, P(a), by the equation:

$$P(a) = 1 - \exp\left(-\int_0^a \lambda(a')da'\right)$$

This is a generalisation of the relationship for an age independent force of infection: $P(a)=1-\exp(-\lambda a)$. The equation above was used to estimate the age specific force of infection from serological data in age groups 0 to 4 years, 5 to 9 years, 10 to 14 years, and 15 to 44 years using a maximum likelihood technique[5].

The value derived for the force of infection in adults (15 to 44 years) was used to estimate the average annual number of infections in pregnancy in England and Wales, thus:

Number of infections in pregnancy = Number of births × Proportion of adults susceptible × Force of infection in adults × Duration of pregnancy

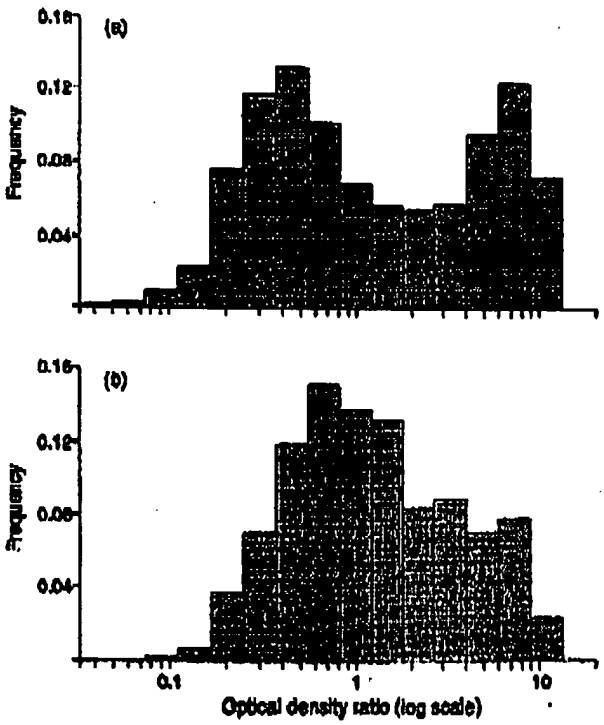
The annual number of births was taken as 700 000 and the duration of pregnancy as nine months.

Results

A total of 6864 specimens of serum was examined. Of these, 1223 were from people aged 1 to 14 years, 3133 from people aged 15 to 29, 1481 from people aged 30 to 44, and 1027 from people aged 45 years or over. Specimens from males numbered 2701 and from females, 4163.

ELISA tests on specimens from one laboratory (Leeds, 636 specimens) yielded different results from specimens from other laboratories. Almost all gave an optical density ratio greater than one. Investigation revealed that Leeds was the only laboratory where specimens were inactivated by heat, and it was thought that this treatment might have invalidated the test results. Consequently, these results were excluded from the analysis. There were no significant differences between the results from the other laboratories, or between the results for males and females.

Figure 1 Distribution of optical density ratios: a) ages 1 to 44 years, b) ages 45 to 99 years.



The optical density ratios for people aged 1 to 44 (5244 specimens) show two distinct peaks, due to positive and negative specimens, but many results fall between the peaks (figure 1a). In the 45 to 99 year age group (984 specimens), no clear division can be seen between positive and negative specimens (figure 1b). This suggests that the assay used does not distinguish specimens with low concentrations of antibody (generally indicative of an infection many years ago) from those with no antibody.

The proportions of positive, negative, and equivocal test results (according to the manufacturer's definitions), in five year age groups, are shown in figure 2. Eight per cent of specimens from 1 to 44 year olds yielded equivocal results. The proportion of equivocal results increased with age, confirming that the test lacks sensitivity in detecting antibody in older people.

Table 1 Estimated force of infection in adults, and annual number of parvovirus B19 infections in pregnancy in England and Wales, using different cut off points

Cut off	Force of infection in adults per year	Infections per 100 000 pregnancies	Average annual total in England and Wales
0.8	0.0081	310	2200
0.9	0.0051	230	1600
1.0	0.0038	170	1200

Figure 2 Antibody prevalence to parvovirus B19 by age

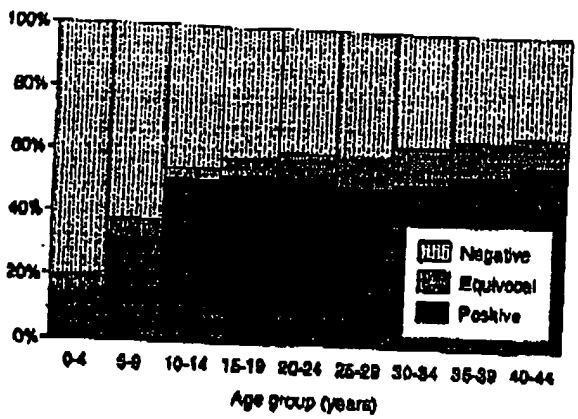
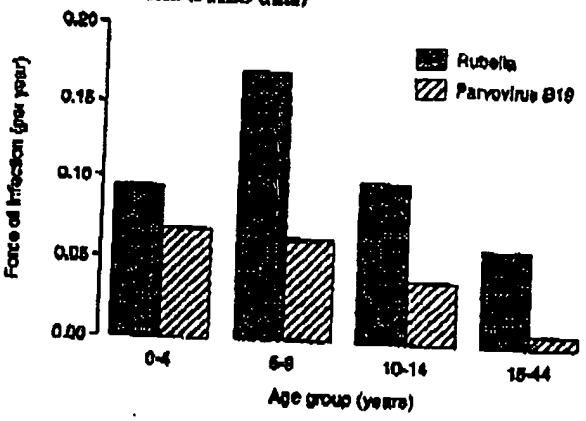


Figure 3 Estimated age specific force of infection for parvovirus B19 (using a cut off ratio of 0.8) and rubella (PHLS data)



Different ELISA cut off points were used to derive the force of infection in adults and estimate the incidence and annual number of parvovirus B19 infections in pregnancy in England and Wales (table 1). The force of infection is greatest in children aged less than 10 years (0.07/year); that is, in a year the probability of a susceptible child under ten becoming infected is 0.07. In adults the force of infection falls to less than 0.01/year. The choice of cut off has only a small effect on the estimated force of infection in children under 15, but has a greater effect in adults. The age specific forces of infection of parvovirus B19 (calculated using a cut off of 0.8) and rubella are compared in figure 3.

Discussion

This serological survey is the most comprehensive yet undertaken for parvovirus B19 and establishes the prevalence of antibody in all age groups. Although the people from whom the specimens were taken were not a random sample of the population, there is no reason to believe that they were unrepresentative in terms of their history of exposure to parvovirus B19. Similar studies of measles, mumps, rubella, and hepatitis A have yielded valuable information about the epidemiology of these diseases[6,7]. Analysis of the serological profile suggests that, on average, slightly fewer than 1% of susceptible adults are infected each year. We estimate from this that 1600 to 2200 mothers are infected in pregnancy each year in England and Wales; that is, approximately one infection in every 400 pregnancies. The total in a particular year will reflect the three to four year epidemic cycle of parvovirus B19: in an epidemic year there may be two or three times more cases than in an average year. An equivalent analysis of serological data for rubella suggests that, before vaccination began, rubella caused about twice as many infections in pregnancy as parvovirus B19.

Our results suggest that the ELISA used was unsuitable for use with serum specimens inactivated by heat, and that its sensitivity was insufficient to detect low concentrations of anti-B19 IgG. A more sensitive test is needed to ascertain the immune status of individuals and would enable the force of infection in adults to be determined more precisely. The test's insensitivity and the changing prevalence of infection with age suggest that an age dependent cut off might improve the analysis by enabling the criterion of equal numbers of false positives and false negatives to be applied in each age group. Methods for determining age specific cut offs are being developed, based on mixture modelling techniques[8].

The force of infection for parvovirus B19 was estimated previously to be 0.004/year in hospital employees and 0.029/year in school employees in a study of seroconversions, over a 42 month non-epidemic period, in Virginia[9]. The risk in hospital workers corresponds well with the overall population estimates from our study, while school employees' higher degree of contact with children puts them at greater risk. Parous women may be at greater risk than nulliparous women for the same reason. This effect has been documented for rubella[10], for which the risk in parous women was two to three times greater than for nulliparous women.

In 1993, an epidemic year for parvovirus B19 in England and Wales, 310 laboratory confirmed infections in pregnancy were reported to the Public Health Laboratory Service Communicable Disease Surveillance Centre. This represents at most 10% of the 3000 to 6000 infections that are estimated to occur in an epidemic year, and this highlights the extent to which B19 infection is undiagnosed. The outcomes of these pregnancies are being studied prospectively in order to provide a better understanding of the consequences of maternal infection during pregnancy.

In a previous study, 180 out of 186 cases were symptomatic and it was estimated that fetal death resulted from maternal infection in pregnancy in 9% of cases, with an excess in the second trimester[3]. Applying this fetal death rate to the estimated number of infections suggests that 150 to 200 fetal deaths are caused by parvovirus B19 each year. It has been suggested, however, that asymptomatic maternal infection is associated with an increased risk of fetal death[11], so this figure could be regarded as a minimum estimate. Symptoms of parvovirus B19, such as rash and arthralgia, are caused by the antigen antibody complex rather than the virus itself. Symptomatic cases may, therefore, have a stronger antibody response than asymptomatic cases, with shorter viraemia and a smaller risk of fetal infection. A study to measure the frequency and consequences of asymptomatic infection in pregnancy is needed.

Parvovirus B19 is an important cause of fetal mortality in the second trimester. Testing for evidence of recent B19 infection should be considered for otherwise unexplained cases of fetal hydrops in the second trimester, especially in epidemic years. The appropriate serological tests on the

mother would include B19 specific IgM and IgG. IgM may, however, not be detected since fetal death associated with parvovirus B19 often occurs two or more months after maternal infection, when B19 IgM has fallen to concentrations difficult to detect with currently available assays. It is important, therefore, to compare IgG in current and antenatal booking specimens, looking for seroconversion to diagnose gestational infection with parvovirus B19.

Diagnosis of fetal infection with parvovirus B19 by the detection of IgM is also of limited value. The most effective method for diagnosing fetal infection is by direct observation of the virus using electron microscopy[12], and/or by parvovirus B19 DNA and antigen assay[13]. These techniques enable fetal viraemia to be detected in pre-natal blood specimens. Infection can also be demonstrated in amniotic fluid or fetal tissues collected at necropsy. Fresh or formalin fixed specimens are suitable for DNA testing, and fetal liver (with a high concentration of the target erythroid cells), is the most useful tissue for confirming the infection. Placenta is not helpful for this investigation.

Although a vaccine against parvovirus B19 is being developed[14], there is no immediate prospect of its use. Greater knowledge of the morbidity and fetal mortality caused by this virus is needed, so that appropriate immunisation strategies may be developed.

Acknowledgements

We thank the staff in the seven public health laboratories for supplying the sera.

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Serological surveillance in the Netherlands

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The Netherlands Immunisation Programme (NIP) has greatly reduced the incidence of target childhood diseases and their complications. The current programme and coverage are shown in the table. Particular vigilance is needed, however, in communities where a high proportion of people are unvaccinated and herd immunity may not be sufficient to protect those who are not immune. Vaccination reduces the circulation of microorganisms, with paradoxical effects: infections occur later in life and may have more complications, natural immunity may not persist because rechallenge occurs less frequently, and immunity in the elderly may decline. Surveillance of immunisation programmes should include the following components: disease incidence, adverse events following vaccination, vaccine efficacy, coverage, and serological surveillance.

A recent pilot study by the National Institute of Public Health and Environmental Protection, in cooperation with the public health services in four municipalities in Utrecht, investigated the feasibility and the management of setting up a serum bank. Factors considered in the protocol included randomisation, assessment of the characteristics of non-